UCRL-11788

University of California

Ernest O. Lawrence Radiation Laboratory

KINETIC STUDIES OF THE TWO LIGHT REACTIONS IN PHOTOSYNTHESIS

Berkeley, California

Submitted for publication to
Photochem. and Photobiol.

UCRL-11788

UNIVERSITY OF CALIFORNIA

Lawrence Radiation Laboratory
Berkeley, California

AEC Contract No. W-7405-eng-48

KINETIC STUDIES OF THE TWO LIGHT REACTIONS IN PHOTOSYNTHESIS

I. D. Kuntz, Jr. and Melvin Calvin

November 1964

UCRL 11288

KINETIC STUDIES OF THE TWO LIGHT REACTIONS IN PHOTOSYNTHESIS

I. D. Kuntz, Jr.* and Melvin Calvin

Lawrence Radiation Laboratory, Laboratory of Chemical Biodynamics** and Department of Chemistry, University of California, Berkeley, California

Abstract--The decay kinetics of the photo-induced absorbance changes in red and green algae are very sensitive to the wavelength of the actinic light. A four to tenfold increase in half-decay time is noted in going from short wavelength (550-650 mμ) to long wavelength (> 700 mμ) excitation. The slow decay rates produced by long wavelength light can be enhanced with a steady background of short wavelength light. A relationship between initial decay rates and O_2 evolution rates is described. This relationship allows a direct correspondence between these spectroscopic studies and the "red-drop" and "enhancement" experiments of Emerson.

---

*National Science Foundation Fellow, 1961-64.

**This work was sponsored, in part, by the U. S. Atomic Energy Commission.

INTRODUCTION

The presence of more than one photochemical reaction within the photosynthetic mechanism has been postulated since the enhancement experiments of Emerson (13). A variety of physical and chemical measurements related to photosynthetic activity have supported this picture of interacting light-driven reactions (21,9,16,26,15,22). In recent years such experiments have been carried down to the spectroscopic level. The photo-induced absorbance changes associated with chlorophyll (20), cytochromes (10,33,27), and quinone (1) have revealed that the steady-state level of these reactive intermediates is closely associated with the color of the actinic light being employed. In general, alterations in the concentrations of reactive species would be expected to result from differences in the rise and/or decay kinetics associated with the photoreactions. The present paper describes experiments designed to study changes in the kinetic patterns associated with various wavelengths of actinic illumination. Preliminary results of some of these studies have been presented earlier (5).

EXPERIMENTAL

Materials. Chlorella pyrenoidosa was grown in continuous culture as described in Ref. 2. Chlorella ellipsoidea, Scenedesmus obliquus,

and Porphyridium cruentum were grown in cotton-plugged 125 ml flasks. Chlorella was grown on modified Meyers' medium (4), Scenedesmus on Lynch's medium (3), and Porphyridium on artificial sea water. Cultures were normally used resuspended in growth medium to an optical density of 1.0 - 1.5 in the 680 chlorophyll band. Because of the different growth conditions and growth rates, no particular care was taken to use cultures of the same age. However, the experiments described below were reproducible from culture to culture regardless of age.

Chloroplasts were prepared by standard techniques (31). Dichlorophenyldimethylurea (DCMU) was purchased from duPont de Nemours, Wilmington, Delaware. 2,6-Dichlorophenol-indophenol (DCPIP) was purchased from K & K Laboratories, Jamaica, N. Y.

Instrumental. The spectrometer employed for the light-dark absorbance changes has been described in detail elsewhere (24). It employs a continuous averaging technique (18) to achieve high sensitivity (10^{-5} optical density units) as well as fast time response (10^{-4} seconds). A block diagram of the spectrometer is shown in Figure 1. The analyzing beam of the spectrometer was produced by a 500 mm Bausch and Lomb monochromator equipped with a 500 watt tungsten projection bulb. We used a 1 mm slit (3 mμ bandwidth). The light intensity was kept as low as feasible by controlling the source voltage. As shown in the figure, the analyzing and actinic beams are at right angles, illuminating a four-sides-clear 1 cm quartz cuvette. One actinic beam was formed by a similar Bausch and Lomb monochromator operated with 3-5 mm slits giving a 10-15 mμ actinic bandwidth. We used a 150 watt tungsten lamp as a second actinic source. In some experiments it provided a continuous "background" light which was focused on the same face of the

sample cuvette as the first actinic beam. Narrow band interference filters (Baird-Atomic) and suitable Corning color glasses were used to select the desired wavelengths. The photomultiplier tube was protected from the actinic light with complementary filters. Typical filter half-band widths were 5-10 mμ.

Relative light intensities were measured with a silicon cell (Hoffman Type 120 CG). Maximum incident intensities were of the order of 10^{16} quanta/sec/cm^2.

The averaging technique requires a repetitive signal. This was conveniently provided by modulating the monochromator actinic light by means of a shutter (phosphor bronze) driven by a commercial stepping motor (Cedar Engineering, Minneapolis, Minn., Model SS-1100). This motor moves in 45° steps on application of a suitable trigger pulse. Transit times are 3 milliseconds. The circuitry was arranged so that successive trigger pulses drove the motor alternatively clockwise and counterclockwise. Thus the shutter first blocked the actinic light beam and then, on a trigger, it moved rapidly to an "open" position. A second trigger pulse restored the shutter to its original closed position. Commercial pulse generators were used (Models 161 and 162, Tektronix, Beaverton, Oregon).

The shutter provides 0.5 - 2.0 millisecond rise or decay times (depending on beam geometry and the driving current supplied to the motor). Jitter is less than 1 millisecond. The "light on" and "light off" times can be independently adjusted to have durations from a few milliseconds to indefinitely long. Because it has a small number of moving parts, the stepping motor should have a long life. It has been

used for many thousands of operations without noticeable change in performance. The drive circuit is shown in Figure 2. It was constructed locally, closely following the manufacturer's suggested circuit.

Errors. The reproducibility of the data in these experiments is limited by coherent noise, low frequency noise, and changes in the biological material. In practice, proper triggering and the use of stable power supplies place the electronic noise level well below the "biological noise". Typical limits on the day-to-day reproducibility of magnitude and rate data are ±10% and ±30% respectively for samples of comparable preparation and treatment. Undoubtedly, variations in the physiological state of the organisms are principal components producing these fluctuations and will ultimately require specific investigation. There is the presumption that these fluctuations do not reflect day-to-day alterations in the basic quantum conversion apparatus. This presumption is, of course, open to experimental test.

RESULTS

I. Decay Kinetics as a Function of Excitation Wavelength

Our first experiments were designed to measure the rise and decay times of the major absorbance change bands as a function of the wavelength of the actinic light. Figures 3A,B and 4A,B show typical absorption spectra and light-dark difference spectra for Chlorella and Porphyridium cruentum.* No background illumination (except for that provided by the weak analyzing beam) was used. Actinic flashes were bright enough to saturate the absorption changes at each actinic wavelength up to 710 mμ. We found that, in general, the rise kinetics were insensitive to exciting

*See also References 33, 7, 19, 10.

wavelength.* On the other hand, the decay kinetics of the absorbance changes showed a pronounced and uniform dependence on the color of the actinic beam, quite apart from any effects of intensity. Excitation with light of wavelengths longer than 700 mμ, which we will henceforth call "$h\nu_1$", produces much slower decay kinetics than does short wavelength actinic light, "$h\nu_2$" (550-630 mμ for Porphyridium; 600-680 mμ for Chlorella and Scenedesmus). Some typical examples of these kinetic effects are shown in Figure 5. "Action spectra" which plot reciprocal half life or initial decay rate as a function of exciting wavelength are given in Figures 6 and 7. In the case of Porphyridium (Fig. 7) the action spectrum indicates that light absorbed by the chlorophyll *a* and that absorbed by the phycobilins participate to different degrees in the reaction studied, an observation well-documented by other types of experiments (12). Table I summarizes the time for half decay for the absorbance changes we have studied to date. We conclude that the initial decay rates of the absorbance changes are a strong function of exciting wavelength; the rates fall sharply in the region of 700 mμ.

II. Effect of Background Illumination on Decay Kinetics

In view of the relatively slow decay rates produced by $h\nu_1$, an obvious experiment is the effect of a background of $h\nu_2$ on the kinetics produced by a flash of $h\nu_1$. This experiment is a logical analog to the Emerson "Enhancement" experiments (13). We find, as Emerson did with O_2 evolution, that the slow decays associated with $h\nu_1$ excitation are speeded up in the presence of a continuous background illumination of $h\nu_2$. The most pronounced effect was seen with the cytochrome bands in Porphyridium (Fig. 8). Our preliminary experiments suggest that the time for

*The few exceptions to this statement will be discussed in a separate ...lication (23).

Table I

Decay Kinetics as a Function of Actinic Wavelength

Organism	Detecting Wavelength (mμ)	Assignment of Absorbance Change*	Time for half decay (sec) $h\nu_2$ excitation	Time for half decay (sec) $h\nu_1$ excitation
Porphyridium	340	Reduced PN	.2	(.3 - .7)
	405	Oxidized cytochrome (f?)	.01$_5$	.26
	422	Reduced cytochrome (f?)	.01	.16
Chlorella elp.	405	Depends on excitation wavelength** (see text)	(.05)**	(.28)**
	425	Chlorophyll, cytochrome (?) (see Ref. 33)	.02	.21
	430	Chlorophyll	.02	.21
	620	? (see Ref. 22)	.07	.28
Chlorella pyr.	340	Reduced PN	.07	.30
	425	Chlorophyll, cytochrome (?) (see Ref. 33)	.07	.36
	432	Chlorophyll	.09	.24
	475	Quinone complex (32); carotenoid (6) chlorophyll b (??)	.08	.25
	520	"	.07	.28
	560	Cytochrome (?)		
	650	Chlorophyll b (??) (see Refs. 20,22)	.05	.16
Scenedesmus	520	Quinone complex (32); carotenoid (6)	.05	.16

*This list reflects the current literature but is not intended to provide "definitive" assignments. Aside from the specific works cited, the reader is referred to Refs. (9),(20) for general discussions.

**The signal polarity is a function of excitation wavelength (see Refs. 23, 33). Hence these time constants may reflect different compounds and hence may not be directly comparable.

half decay for the 405 and 422 mμ bands is inversely proportional to the background intensity. A similar but smaller effect was measured for the 520 mμ band in Chlorella and Scenedesmus. Fork has recently reported qualitatively similar results for the 590 mμ absorbance change in Chlorella and in Ulva (8). Rumberg has also reported systems in which decay rates were enhanced by using a second flash of light in place of a continuous background (29).

GENERAL REMARKS

Effects of DCMU. Excitation with either $h\nu_1$ or $h\nu_2$ in the presence of 10^{-5} M DCMU yields similar kinetics and signal magnitudes to those produced by $h\nu_1$ in the unpoisoned system (Fig. 9).

Chloroplast and Quantasome Preparations. Spinach chloroplasts and quantasomes (28) were studied under a variety of reaction conditions. These included untreated fresh chloroplasts and leaf homogenate, chloroplasts in Hill reaction mixtures (ferricyanide or DCPIP) and triphosphopyridine nucleotide (TPN) reduction systems, using either H_2O or DCPIP-ascorbate as electron donor (31). None of the broken leaf fractions showed rise or decay kinetics that were influenced by the color of the actinic light (Fig. 10), although sections of the whole leaves from which they were made showed the general behavior reported for green algae.

Intensity Dependence. A detailed study of the light intensity dependence of the absorbance change kinetics is in preparation. The results of interest here are: (1) rise and decay kinetics in these green and red algae show some intensity dependence, the initial rise rates are directly related to intensity (not necessarily linearly); (2) the effects described in this paper are most pronounced at high actinic intensities; (3) taken as a whole, these intensity effects are not sufficient to explain the

results ascribed to actinic wavelength.

DISCUSSION

It is interesting to note that, regardless of the detailed mechanism involved, much of the data presented in this paper can be conveniently summarized in terms of the Emerson "red-drop" and "enhancement" experiments on O_2 evolution (14,13). To make this comparison, we need only assume that the rate of O_2 evolution is directly related to the steady-state rate of electron flow through a series of reversible redox reactions. We can convert our kinetic data into rates of electron flow if we measure the initial rates of the dark reactions. A detailed discussion of the assumptions required to relate rates of O_2 evolution to the reaction rates of electron transport intermediates is given in the appendix. These assumptions are not particularly restrictive, and would be consistent with most proposed mechanisms for photosynthesis.

Thus, we interpret the drop in initial decay rates observed at long excitation wavelengths (Figs. 5, 6, 7, Table I) as reflecting the same phenomenon that Emerson observed when he found that the rate of O_2 evolution in far-red light was much slower than in light of shorter wavelength. Furthermore, we feel the increase in the "far-red" initial decay rates when a short wavelength background light is added (Fig. 8) is closely related to the "enhancement" of O_2 evolution rates in similar experiments. Finally, the slow decay rates observed for DCMU-treated algae (Fig. 9) are consistent with the marked inhibition of O_2 evolution in these cases. We feel that the changes in decay rates involved are large enough to explain the alterations in the steady-state concentrations of photoactive intermediates mentioned in the introduction (20,10,33,27,1,21).

This is particularly true of Porphyridium where the "enhancement" in decay rates can be quite striking.

The correspondence with the Emerson experiments lends support to two of our major conclusions:

(1) Two or more light-driven reactions have been demonstrated at the spectroscopic level in terms of kinetic behavior of the optical density changes absorbance.

(2) These light reactions can interact with one another.

Our study can also point to the mode of the interaction between the two light reactions:

(3) Generally speaking, the products of the light reaction interact with each other at the level of "dark" recovery reactions. This follows from our primary observation that the major difference between "$h\nu_1$" and "$h\nu_2$" excitation is a difference in decay rates rather than rise rates. An even more direct demonstration of the type of interaction arises from the "two-light" experiments where there was a speed-up in the decay reactions produced by $h\nu_1$ when $h\nu_2$ was added as background light.

(4) The whole cell environment seems to be required for the efficient interaction of the two-light systems because chloroplast preparations which were physiologically active for $TPNH_2$ formation or Hill reactions did not show any of the whole cell effects described above.*

The conclusions reached here concerning the photosynthetic mechanism have, for the most part, been reached independently by other workers using many different techniques. Observations of these types are most frequently used to support the broad outlines of the Hill-Bendall mechanism (17)

---

*Rumberg (29) has reported a specially treated chloroplast system which does show spectroscopic interactions of two light reactions. The reasons for the difference between his system and ours are not clear.

which postulates two light reactions cooperating in series with each other. Rather than developing this point of view further, we will discuss briefly two aspects of this work which require some effort to fit into the Hill-Bendall picture.

First, in connection with the enhancement experiment shown in Fig. 8, it should be pointed out, [illegible], that the fastest decay time observed in the double irradiation experiment (40 milliseconds) is considerably longer than the 10-20 millisecond decay produced by 560 mμ excitation alone (Fig. 5). Furthermore, the absorbed intensity of 560 mμ light used as background illumination in this experiment was fifty times that of the 700 mμ flash. Thus, there does not seem to be a simple quantum-for-quantum interaction between red and green light. The simplest explanation consonant with the series mechanism is that $h\nu_2$ drives both light reactions approximately equally, thus leaving very few excess quanta to couple with the flash of $h\nu_1$. An alternative explanation is that the coupling of the two light reactions represented by this enhancement of decay rate is, in fact, an inefficient process.

Second, to pursue this latter argument a bit further, the chloroplast preparations described in the text showed good quantum yields for, say, $TPNH_2$ reduction with H_2O. But they showed none of the kinetic effects ascribed to the presence of two cooperating light reactions, nor did they show significant "enhancement" effects as measured by $TPNH_2$ reduction rates (30). These observations might lead us to conclude that efficient photosynthesis could well require only one photochemical reaction.

In conclusion, although the correspondence described in this paper between the spectroscopic results and the oxygen evolution experiments is

quite encouraging both as a tool for further study and as evidence that the absorbance changes are rather directly related to the photosynthetic processes, it should be remembered that the molecular basis for, and the importance of, the Emerson enhancement effects is still unknown.

Appendix

Consider an electron transport pathway which transfers electrons from H_2O to CO_2. This pathway can include one or more photochemical reactions as well as a number of reversible redox couples. Assume that there are no parallel alternative electron paths.

Let us apply two restrictive conditions, derived from experiment, to this system.

(1) After a sufficient period of illumination, the system comes to steady-state.

(2) At steady-state, the flow of electrons into the system (measured by O_2 evolution) is equal to the flow of electrons out of the system (CO_2 uptake).

The first condition requires the intermediate concentrations to be constant, that is, at steady-state $\frac{dx}{dt} = 0$, where x = any intermediate. Another way of expressing this restriction is to say that the sum of all reaction rates tending to create x will just balance the sum of all reaction rates tending to destroy x.

The second condition asserts that at steady-state the net electron flow anywhere in the non-branching system is constant. From this condition, when the system is at steady-state, we can assert

$$\Sigma \frac{dx}{dt} \text{ production} = \Sigma \frac{dx}{dt} \text{ destruction} = \text{electron flow rate} \quad \text{(Eqn. 1)}$$

Thus, to measure the rate of electron flow, we need either the steady-state production rate or the steady-state destruction rate of the electron transport intermediates. Since under steady-state conditions there is no net concentration change of any intermediates, our spectroscopic methods cannot directly measure these rates.

However, for those intermediates directly involved in a photochemical reaction, the cessation of illumination will drop the production rate to zero as fast as illumination is turned off. Thus, a measure of the initial rate of the destruction reaction will tell us the steady-state electron flow rates and thus, for these intermediates, the initial rate of the dark reaction should be equal to the steady-state rate of O_2 evolution. The same analysis holds for any intermediates connected to the primary quantum conversion products by reactions which are fast with respect to the rate of quantum input and to the rate at which the light is turned off.

Our measurement of initial decay rates is also justified for those cases where the decay rates are proportional to the electron flow rates rather than the equality described above (Eqn. 1).

Generally speaking, the assumptions involved here do not impose severe restrictions on a photosynthetic mechanism. These mechanisms are consistent with most of the proposed mechanisms for photosynthesis (Refs. 20,25,11) and allow us to draw a direct correspondence between our spectroscopic results and the "red-drop" and "enhancement" experiments of Emerson.

REFERENCES

1. J. Amesz, Biochim. et Biophys. Acta 79, 257 (1964).
2. J. A. Bassham and Melvin Calvin, The Path of Carbon in Photosynthesis, Prentice-Hall, Inc., Englewood Cliffs, N. J., 1957.
3. D. F. Bradley and M. Calvin, Proc. Natl. Acad. Sci. 41, 563 (1955).
4. J. S. Burlew (ed.), Algae Culture from Laboratory to Pilot Plant, Carnegie Institute of Washington, 1953, p. 94.
5. M. Calvin, I. D. Kuntz, Jr. and R. H. Ruby, Proc. IVth Int. Photobiol. Congress, Oxford, England, 1964, in press.
6. B. Chance and B. Strehler, Plant Physiol. 32, 536 (1957).
7. J. W. Coleman and E. Rabinowitch, J. Phys. Chem. 63, 30 (1959).
8. Y. de Kouchousky and D. C. Fork, Proc. Natl. Acad. Sci. 52, 232 (1964).
9. L.N.M. Duysens, in Photosynthetic Mechanisms of Green Plants, (B. Kok A. Jagendorf, Eds.), p. 1. National Academy of Sciences-National Research Council, Washington, D. C. (1963).
10. L.N.M. Duysens, in Progress in Photobiology, (B. C. Christensen and B. Buchmann, Eds.), p. 135. Elsevier Publishing Co., Amsterdam (1961).
11. L.N.M. Duysens, Proc. Roy. Soc. 157B, 301 (1963).
12. L.N.M. Duysens and J. Amesz, Biochim. et Biophys. Acta 64, 243 (1962).
13. R. Emerson, R. Chlamers and C. Cederstrand, Proc. Natl. Acad. Sci. 43, 133 (1957).
14. R. Emerson and C. M. Lewis, Am. J. Bot. 30, 165 (1943).
15. C. S. French, Ref. 9, p. 355
16. J. C. Goedheer, Biochim. et Biophys. Acta 66, 61 (1963).
17. R. Hill and F. Bendall, Nature 186, 136 (1960).
18. M. P. Klein and G. W. Barton, Rev. Sci. Instr. 34, 754 (1963).

Figure 1. Block diagram of spectrometer.

Figure 2. Drive circuit for stepping motor. External trigger pulses are required, see text.

Figure 3A,B. Absorption spectrum and light-dark difference spectrum for Chlorella cells. The absorption spectrum was obtained in a Cary 14M spectrometer equipped with a Model 1462 scattered transmission attachment. The difference spectrum was obtained using saturating red (600-720 mμ) light except for dotted portions which required special filter combinations.

Figure 4A,B. Absorption spectrum and light-dark difference spectrum for Porphyridium cruentum cells. Conditions as described in legend for Figure 3A,B.

Figure 5. Representative decay kinetics induced by different actinic wavelengths.

Figure 6. Reciprocal times for half decay as a function of actinic wavelength. 520 mμ absorption change in Chlorella. Part of the absorption spectrum is shown for reference. Maximum value about 15 sec^{-1}.

Figure 7. Relative initial decay rates as a function of actinic wavelength. 422 mμ absorption change in Porphyridium. Part of the absorption spectrum is shown for reference.

Figure 8. "Enhancement experiment". Flash 710 mμ, Background 560 mμ. 422 mμ band in Porphyridium.

Figure 9. Effect of 10^{-5} M DCMU on 430 mμ absorbance change in Chlorella.

Figure 10. Absorbance change kinetics in various broken cell reaction mixtures. Preparation of materials and reaction conditions are described in Ref. 43.

SPECTROMETER —— BLOCK DIAGRAM

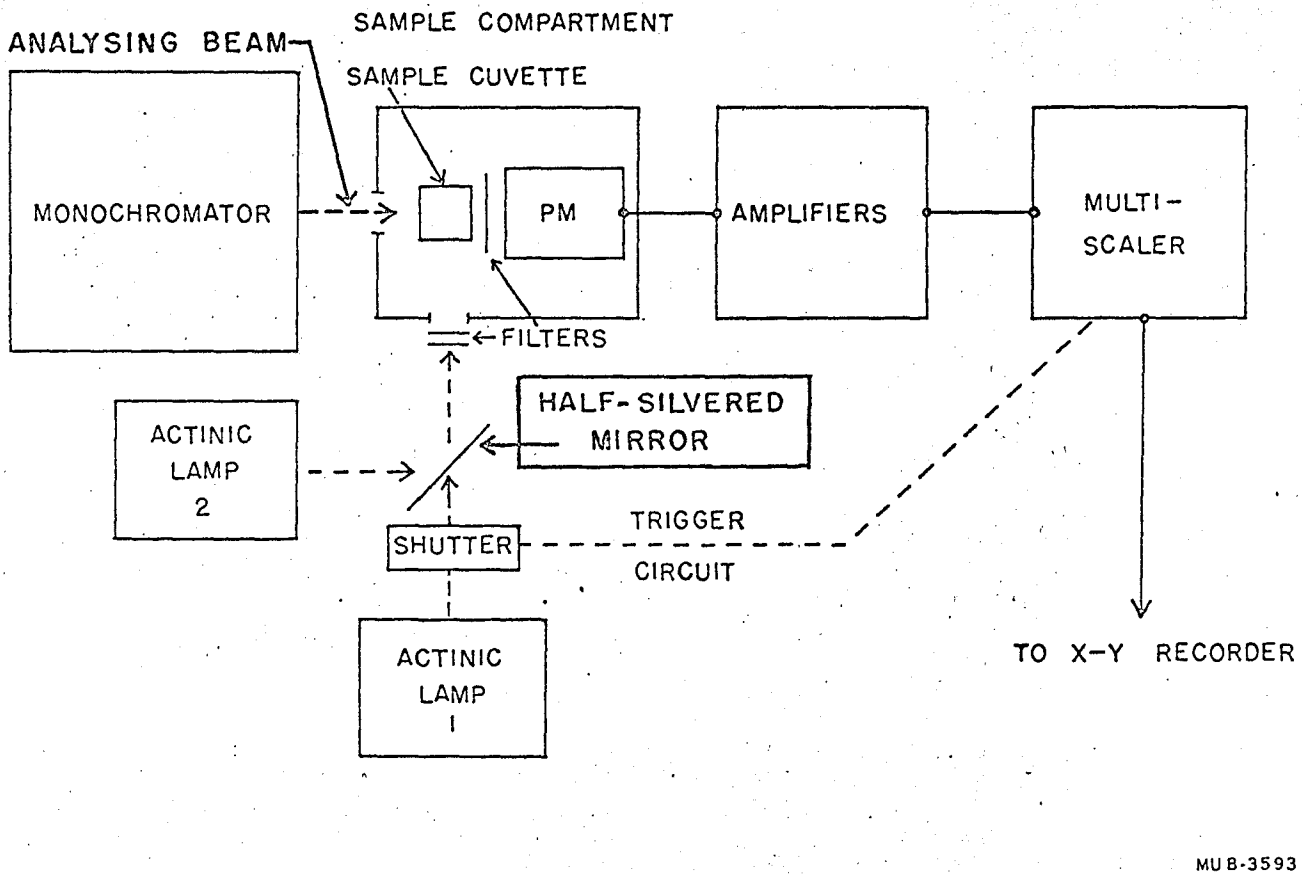


Fig. 1

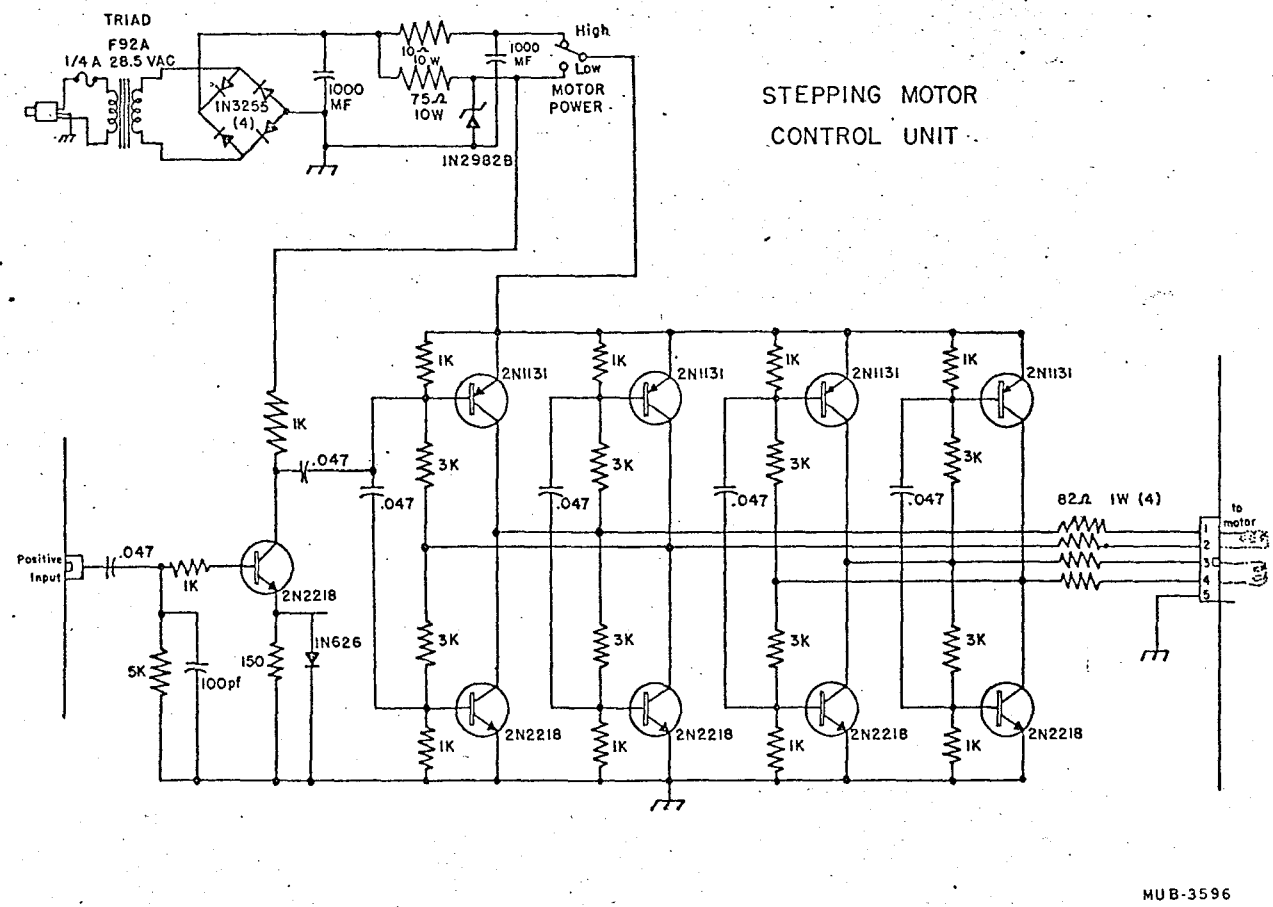

TRIAD
F92A
1/4 A 28.5 VAC
1N3255
(4)
1000
MF
10Ω
10W
75Ω
10W
1000
MF
High
Low
MOTOR
POWER
1N2982B
STEPPING MOTOR
CONTROL UNIT
1K
2N1131
3K
.047
82Ω 1W (4)
to
motor
Positive
Input
2N2218
5K
100pf
150
1N626
MUB-3596

Fig. 2

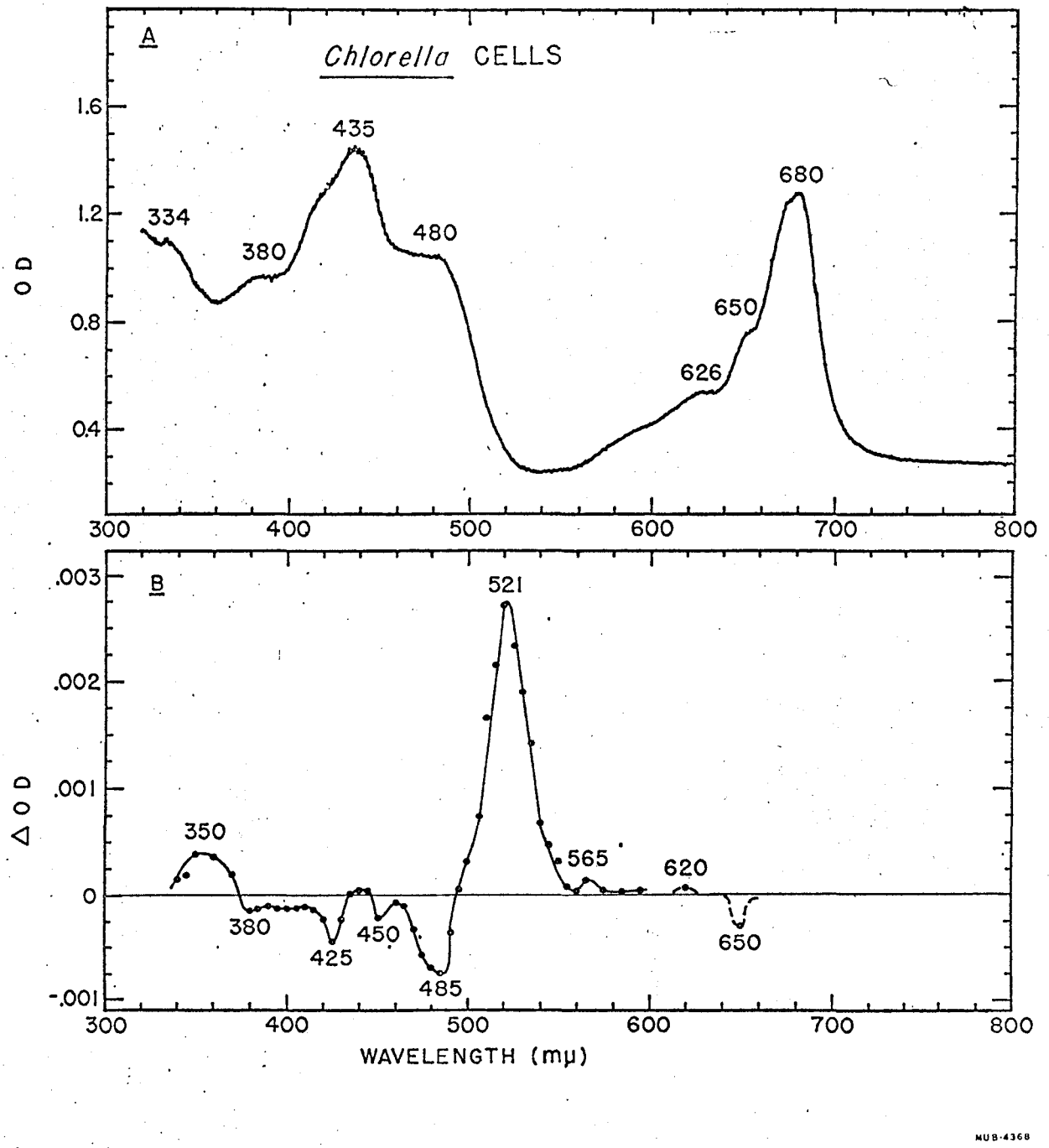

A
Chlorella CELLS
O D
1.6
1.2
0.8
0.4
334
380
435
480
626
650
680
300
400
500
600
700
800
B
Δ O D
.003
.002
.001
0
-.001
350
380
425
450
485
521
565
620
650
300
400
500
600
700
800
WAVELENGTH (mμ)
MUB-4368

Fig. 3A, B

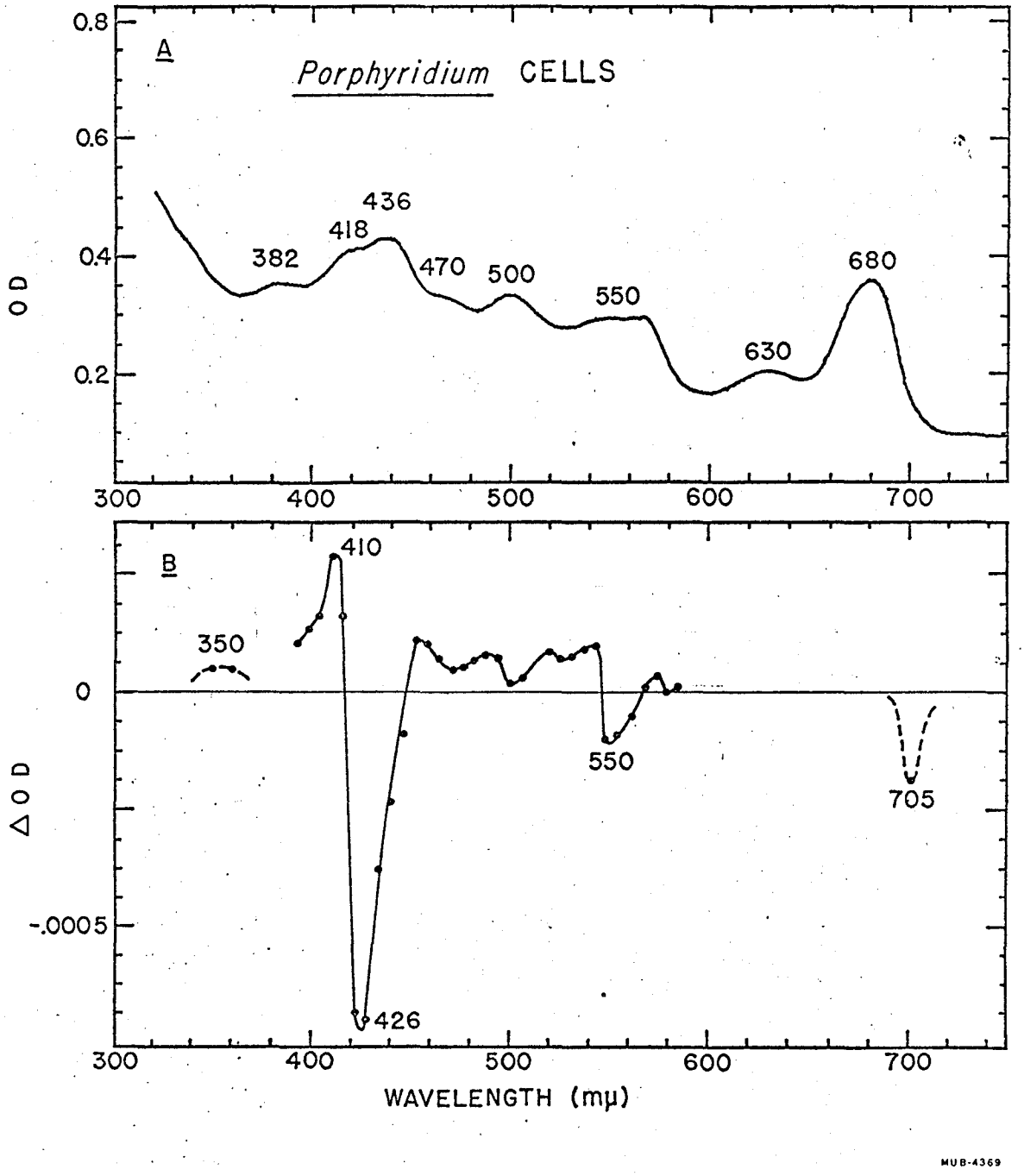

A
Porphyridium CELLS
0.8
0.6
0.4
0.2
O D
382
418
436
470
500
550
630
680
300
400
500
600
700
B
410
350
0
550
705
Δ O D
-.0005
426
300
400
500
600
700
WAVELENGTH (mμ)
MUB-4369

Fig. 4A, B

KINETIC DEPENDENCE ON EXCITATION WAVELENGTH

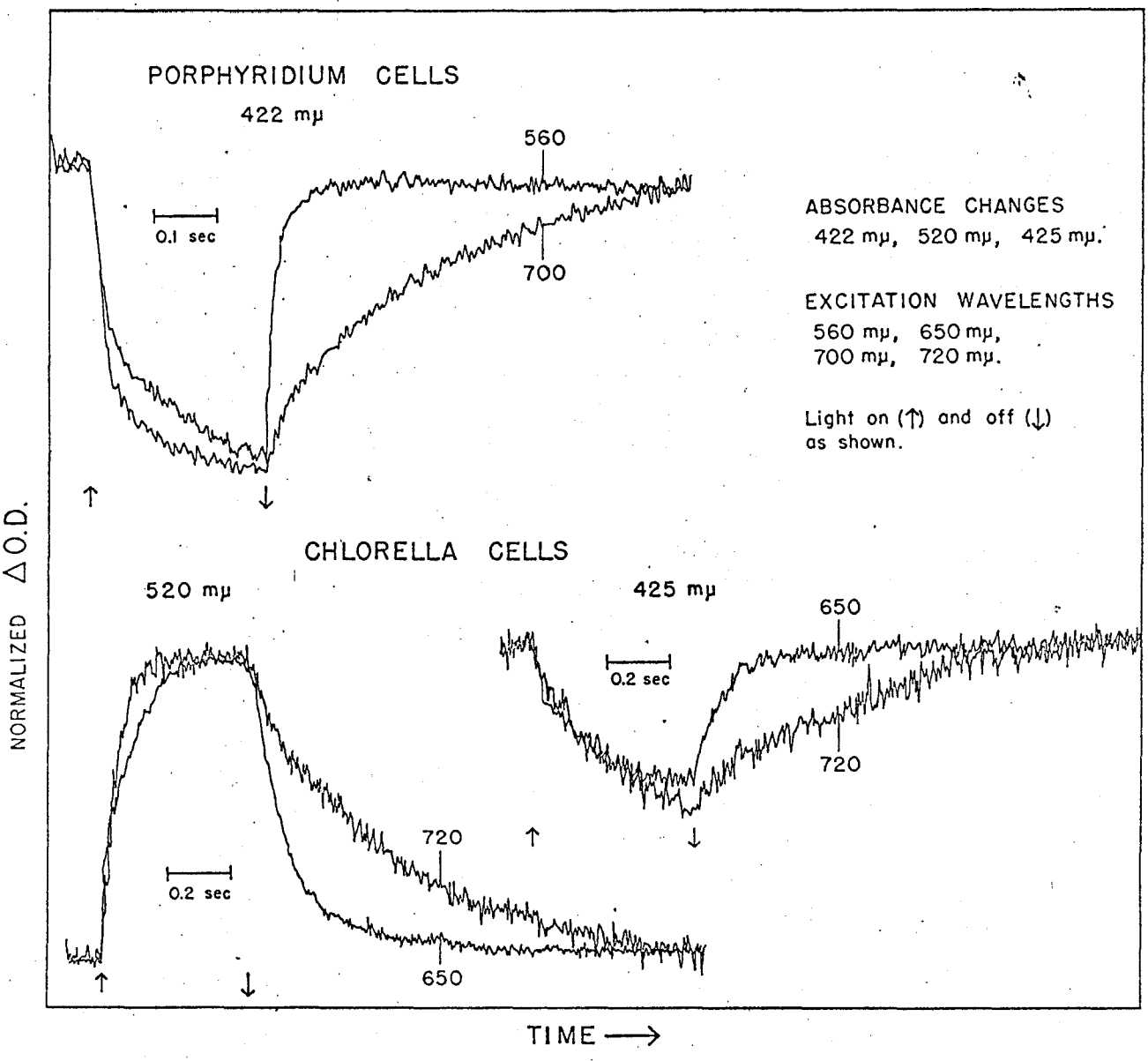


Fig. 5

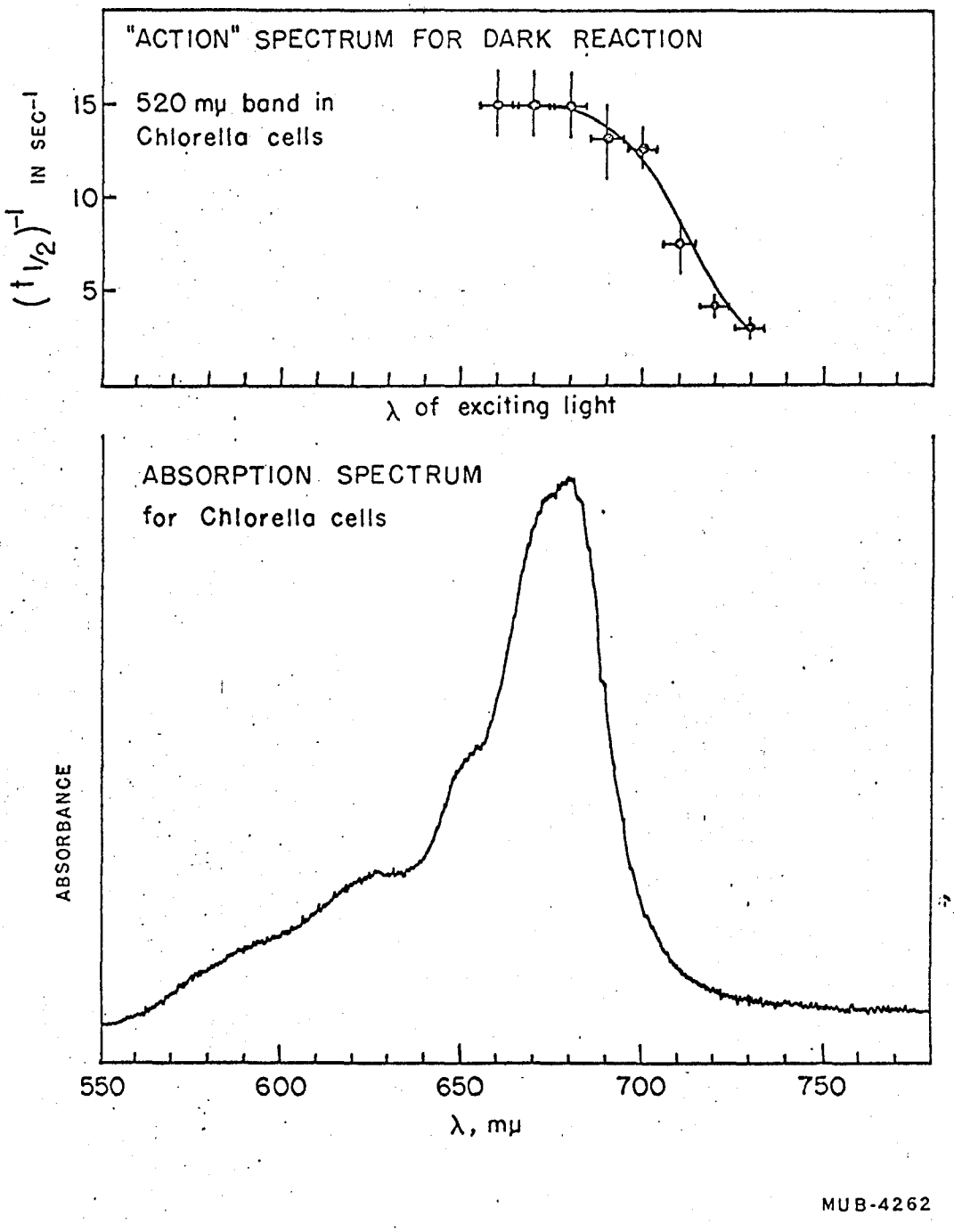

"ACTION" SPECTRUM FOR DARK REACTION
520 mμ band in
Chlorella cells
$(t_{1/2})^{-1}$ IN SEC^{-1}
15
10
5
λ of exciting light
ABSORPTION SPECTRUM
for Chlorella cells
ABSORBANCE
550
600
650
700
750
λ, mμ
MUB-4262

Fig. 6

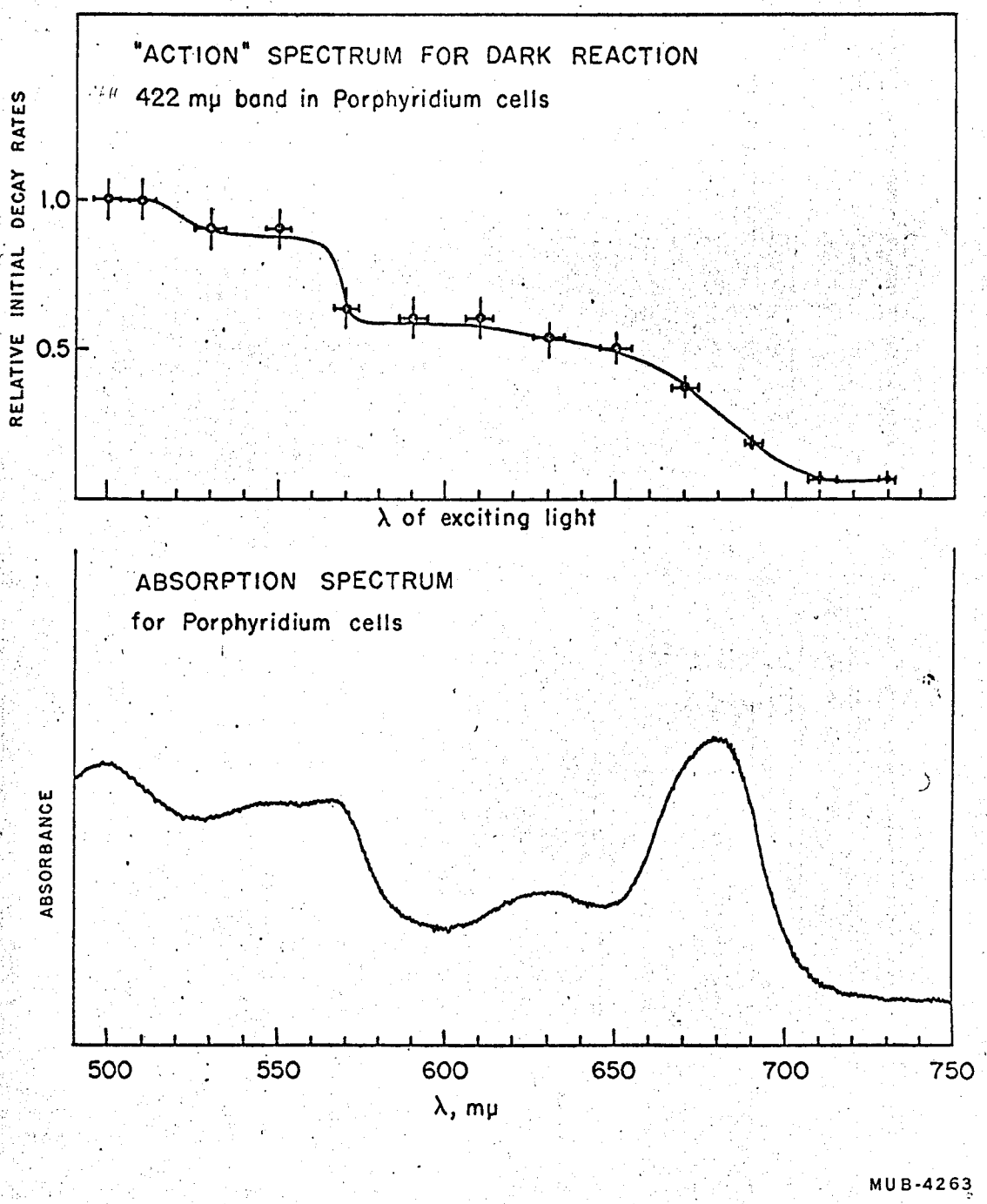
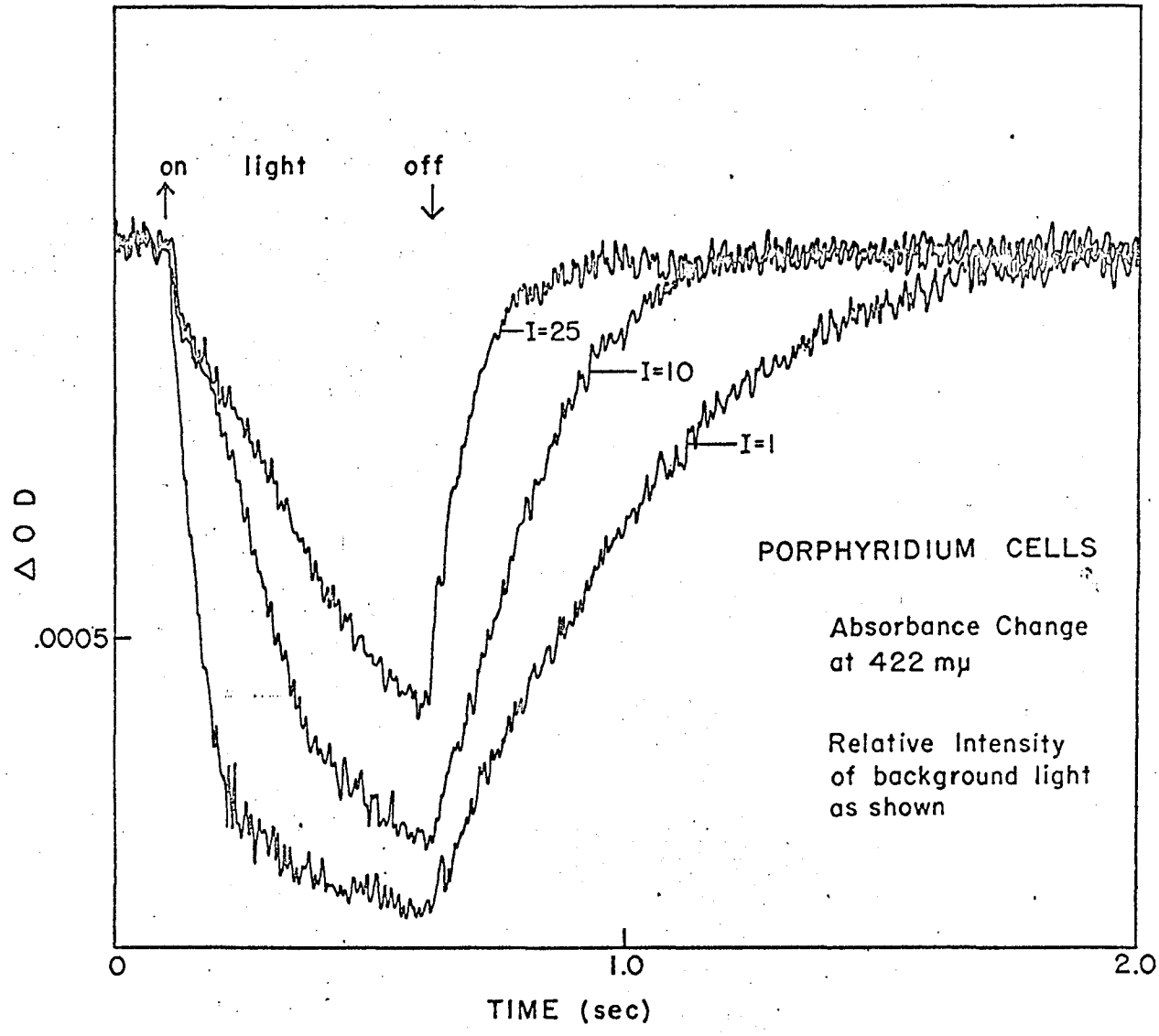

"ACTION" SPECTRUM FOR DARK REACTION
422 mμ band in Porphyridium cells
RELATIVE INITIAL DECAY RATES
1.0
0.5
λ of exciting light
ABSORPTION SPECTRUM
for Porphyridium cells
ABSORBANCE
500
550
600
650
700
750
λ, mμ
MUB-4263

Fig. 7

EFFECT OF BACKGROUND LIGHT (550mμ) UPON KINETICS
INDUCED BY 710 mμ LIGHT



MUB-3591

Fig. 8

EFFECT OF DCMU

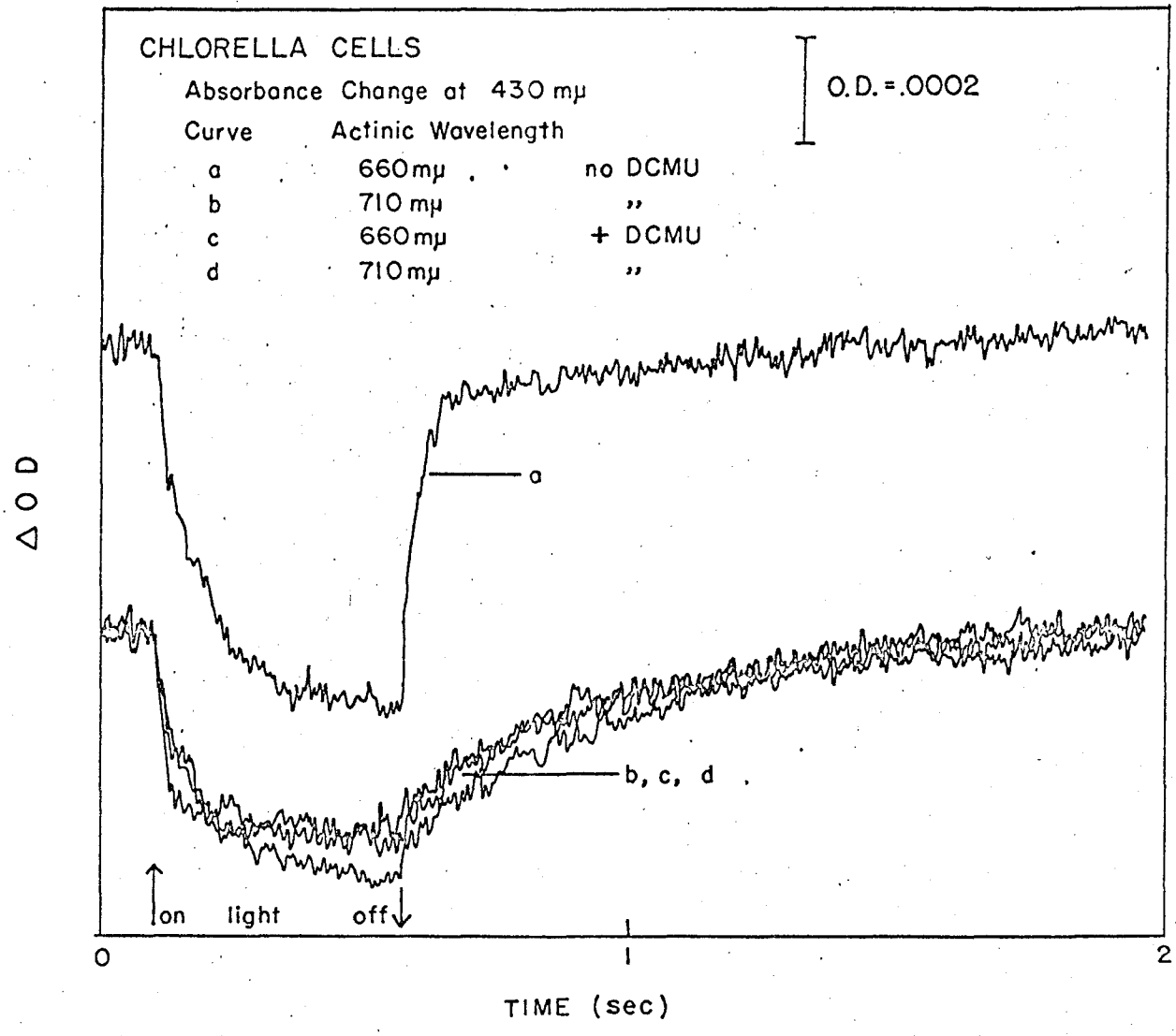


Fig. 9

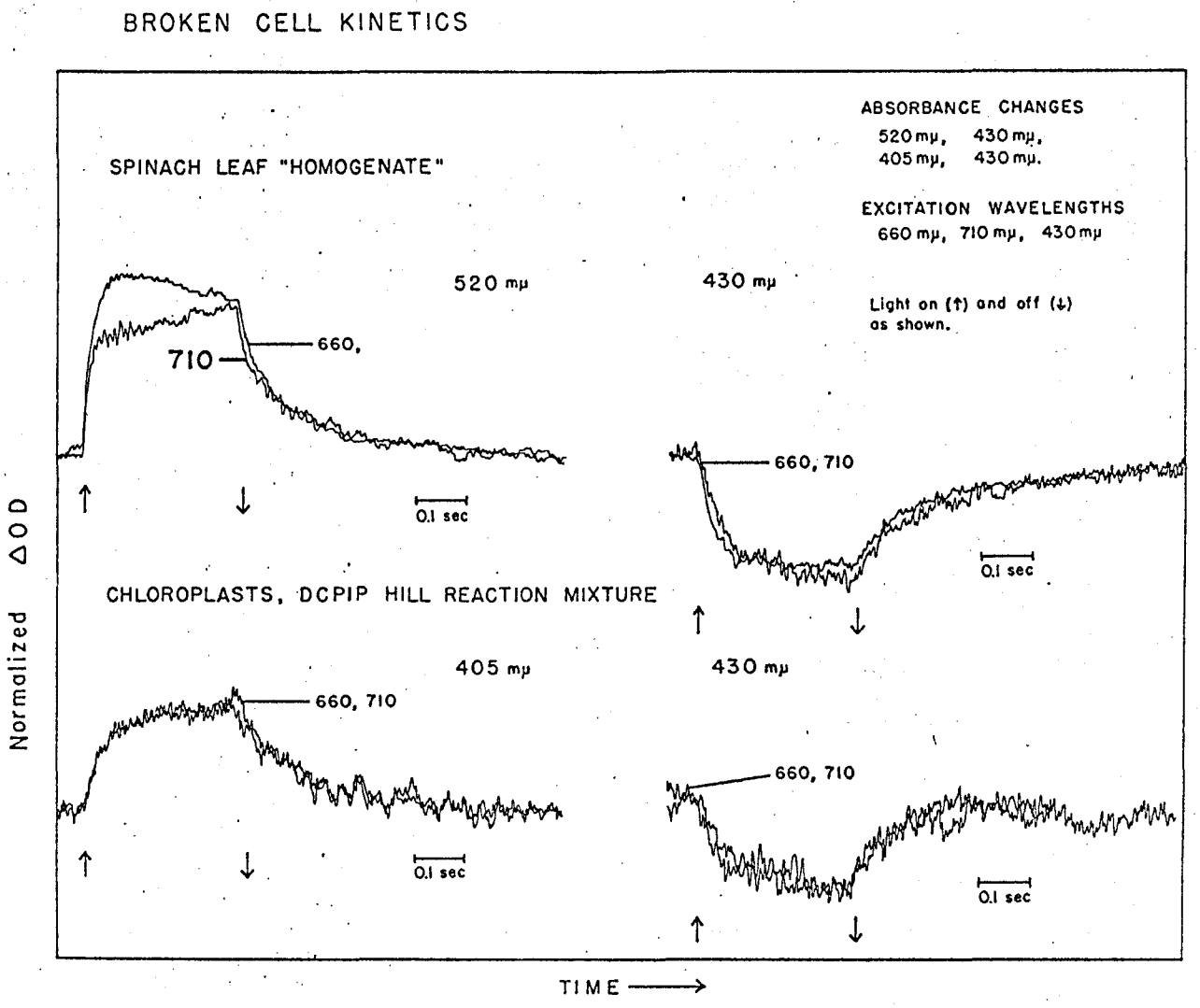

BROKEN CELL KINETICS
ABSORBANCE CHANGES
520 mμ, 430 mμ,
405 mμ, 430 mμ.
EXCITATION WAVELENGTHS
660 mμ, 710 mμ, 430 mμ
Light on (↑) and off (↓) as shown.
SPINACH LEAF "HOMOGENATE"
520 mμ
430 mμ
660,
710
660, 710
0.1 sec
CHLOROPLASTS, DCPIP HILL REACTION MIXTURE
405 mμ
430 mμ
660, 710
660, 710
0.1 sec
Normalized ΔOD
TIME

Fig. 10